REPORT DOCUMENTATION PAGE

AD-A266 249

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	REPORT DATE	REPORT TYPE AND DATES COVERED
		FINAL 1 Dec ?? to 30 Nov ??

TITLE AND SUBTITLE

NEW INVESTIGATOR AWARD

FUNDING NUMBERS

AFOSR-??-????
61102F
2312
A5

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Bolling AFB DC 20332-0001

Dr Kozumbo

10 SPONSORING/MONITORING AGENCY REPORT NUMBER

DTIC
ELECTE
JUN 28 1993
S D E

11 SUPPLEMENTARY NOTES

12a DISTRIBUTION AVAILABILITY STATEMENT

Approved for public release;
distribution unlimited.

12b DISTRIBUTION CODE

13 ABSTRACT (Maximum 200 words)

This one year new investigator award resulted in sequencing the Ah receptor, identifying the ligand binding domain of this receptor and development of a purification scheme that can generate milligram quantities of recombinant Ah receptor in a few days time.

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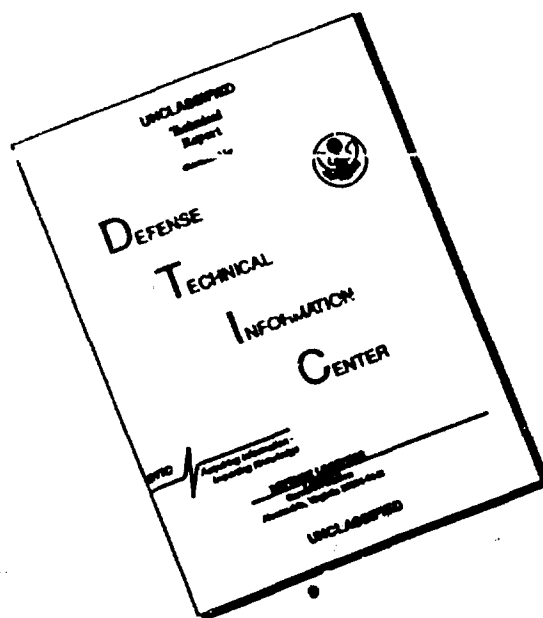| 14 SUBJECT TERMS			15 NUMBER OF PAGES
			16 PRICE CODE
17 SECURITY CLASSIFICATION OF REPORT (U)	18 SECURITY CLASSIFICATION OF THIS PAGE (U)	19 SECURITY CLASSIFICATION OF ABSTRACT (U)	20 LIMITATION OF ABSTRACT (UL)

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Final Summary Report

Molecular Characterization of the Ah-Receptor: Immunoaffinity Purification, Amino Acid Sequencing and Generation of Epitope Specific Antibodies.

Introduction:

The Ah-receptor is a soluble protein which regulates the expression of a number of genes encoding enzymes involved in the metabolism of foreign compounds (e.g.. cytochrome-P450s, glutathione S-transferases, quinone reductases, etc.). In response to potent agonists, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the Ah-receptor also mediates a variety of toxic responses, including epithelial hyperplasia and metaplasia, lymphoid involution, tumor promotion and teratogenesis. Our recent cloning of the Ah-receptor cDNA has demonstrated that this receptor is a member of a newly discovered family of transcription factors characterized by an N-terminal helix-loop helix motif and an adjacent domain responsible for ligand binding (in the Ah-receptor) and with unknown function in other family members (i.e., Sim, Per and Arnt proteins).

Research in my laboratory can be divided in to two major areas of emphasis: 1) understanding the mechanism by which the Ah-receptor mediates the toxicity of halogenated aromatic compounds like TCDD and 2) determining the risk that compounds like TCDD pose to human and wildlife populations. As an initial step in reaching these objectives, we have purified the Ah-receptor to homogeneity, sequenced the proteins N-terminus, raised antibodies against a synthetic peptide corresponding to this sequence, cloned the Ah-receptor cDNA from both mouse and human and cloned and characterized the murine structural gene (the Ah-locus).

Research Accomplishments Under AFOSR New Investigator Grant, 1990.

On July 1, 1990, the AFOSR granted $54,000 in direct costs to fund our research on the Ah-receptor. At that time we had just purified the Ah-receptor and had limited amino acid sequence data. As a result of funding from the AFOSR, we have generated additional amino acid sequence data, identified the ligand binding domain of the Ah-receptor and developed a purification scheme which can generate mg quantities of recombinant Ah-receptor in a few days time.

Specific Aims 1 & 4: Develop an immunoaffinity purification scheme to purify the Ah-receptor in high yield and generate amino acid sequence from internal peptide fragments. Prior to our work under this grant proposal, we had developed a method by which we could purify the Ah-receptor from mouse liver to homogeneity (approximately 200,000-fold). Unfortunately, this method was expensive, time consuming and could only generate limited quantities of pure protein (approximately 5 ug in two weeks time). Therefore, we had proposed to improve our purification, using antibodies raised against a synthetic peptide corresponding to the receptors N-terminus, in an immunoaffinity purification scheme. Early on in the funding period, we found that an alternative strategy might hold more promise. That strategy was to improve our existing purifications scheme,

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generate additional amino acid sequence data from the purified protein, use that amino acid sequence data to clone the receptor's cDNA, and then express the cDNA in prokaryotic and eukaryotic expression systems to generate unlimited quantities of pure receptor (this was described as an alternative strategy in our proposal). To this end, we spent approximately 3 months optimizing our purification scheme and microsequencing techniques. We then purified approximately 50 ug of receptor and subjected it to CNBr fragmentation and sequenced 3 internal peptide fragments. Using funding from other granting agencies, we used this sequence data to clone the mouse and human cDNA. By comparison of the cDNA sequences with the amino acids sequences we generated from pure protein, we were able to identify the ligand binding domain of the receptor, since one of our sequenced fragments was radiolabeled with the photoaffinity ligand [^{125}I]-2-iodo-7,8,-dibromodibenzo-p-dioxin (Figure 1).

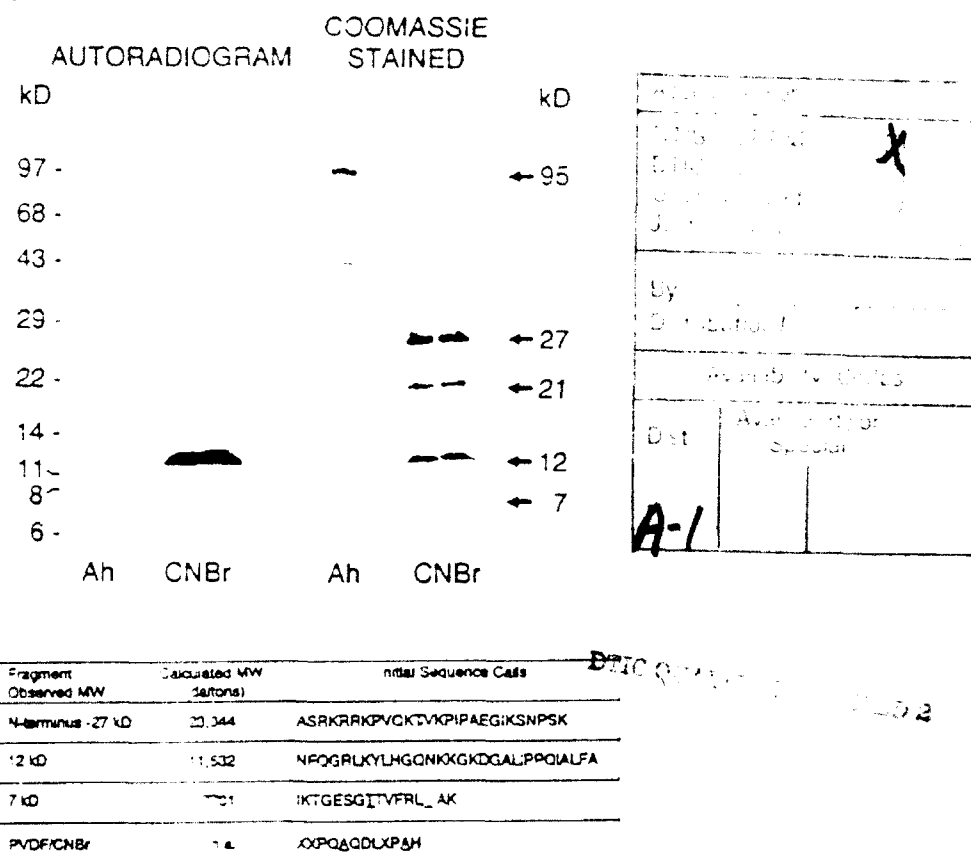| Fragment Observed MW	Calculated MW (daltons)	Initial Sequence Calls
N-terminus -27 kD	20,344	ASRKRRKPVGKTVKPIPAEGIKSNPSK
12 kD	11,532	NFQGRLKYLHGQNKKGKDGALPPQIALFA
7 kD	n.a.	IKTGESGITVFRL_AK
PVDF/CNBr	n.a.	XXPQAQDLXPAH

Figure 1: CNBr cleavage products of the Ah-receptor. (Top) 500 pmol of the purified protein covalently labeled with 125-I-2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin, was dissolved in 100ml of 70% formic acid. CNBr was added and the reaction was carried out at room temperature, in the dark, under nitrogen for 24 hrs. The cleavage products were separated by 12% Tricine-SDS-PAGE, electroblotted onto PVDF membranes, and stained with Coomassie blue dye. The major fragments were subjected to N-terminal sequencing on a pulsed liquid phase sequenator at the Northwestern University Biotechnology Center. Left panel: autoradiogram showing fragment covalently labeled by photoaffinity ligand (12 kD). Right panel: Coomassie blue stained membrane showing size distribution of the CNBr fragments. Uncleaved protein shown in left lanes of each panel. 21 kD fragment is mixture of at least 2 fragments and remains unresolved. (Bottom) Sequence and calculated and observed molecular weights of the individual CNBr fragments. Lower case letters indicate less certain determinations of amino acid identity. PVDF/CNBr refers to fragment generated by cleavage on the PVDF membrane. n.a. = not applicable.

Specific Aim #2: Generate quantities of Ah-receptor for use in immunization protocols in an effort to raise monoclonal antibodies. Once we had the cDNA, we began to develop systems to express this protein in large amounts. This protein can then be used in functional studies and as a reagent to generate monoclonal antireceptor antibodies. We have developed two independent systems for the overexpression of Ah-receptor protein. The first is a bacterial expression system, where the Ah-receptor is fused to the glutathione S-transferase gene. The resulting fusion protein is easily purified by adsorption to a glutathione column. The two proteins can then be separated by taking advantage of a thrombin cleavage site which has been engineered into the protein fusion juncture. Using this system, we are able to generate mg quantities of recombinant Ah-receptor in about one week (figure 2). In an effort to raise monoclonal antibodies against this protein, we have begun screening hybridomas derived from immunized mouse spleen cells. We currently have 5 sets of hybridoma cells to screen (5 mice). Unfortunately, our funding for this project from the AFOSR has been exhausted and this screening step may have to wait until we can find renewed financial resources to complete this project.
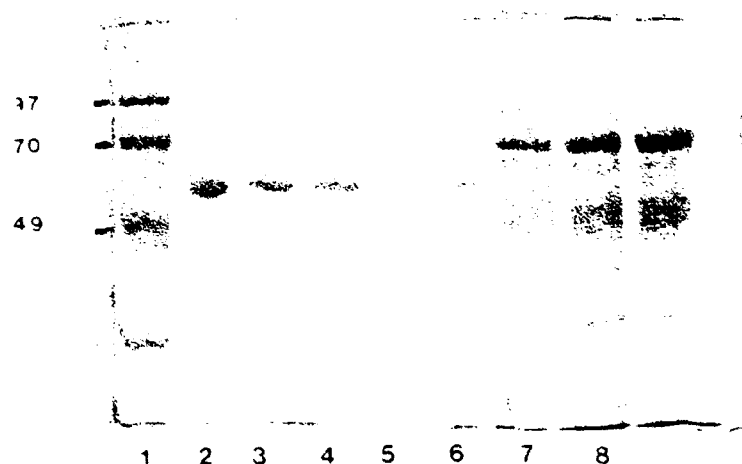

Figure 2: Generation of mg quantities of Ah-receptor fusion protein. The Ah-receptor was cloned into the BamHI site of the vector pGEX2T. The resulting glutathione S-transferase-Ah-receptor fusion protein was produced in e.coli and purified on a glutathione agarose column. Lane 1, molecular weight standards; lane 2, 5 ug BSA; Lane 3, 2.5 ug BSA, Lane 4, 1 ug BSA; Lane 6 0.5 ug BSA, Lane 7, 1/1000 of a 1 liter preparation of the fusion protein; Lane 8, 1/500 of a 1 liter preparation of the fusion protein.

Specific Aim #3: Characterization of monoclonal antibodies raised against the Ah-receptor:

As stated above, we have frozen down hybridomas from 5 mice which were immunized with the recombinant Ah-receptor. Since the AFOSR grant was our only funding for this work, and since those funds are exhausted, we are awaiting funding from another source prior to continuing.

Publications:

Burbach, KM, Poland A and Bradfield, CA (1991). Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proceedings of the National Academy of Sciences (USA). **89**:.

Bjeldanes, LF, Kim, J-Y, Grose, KR, Bartholomew, JC and Bradfield, CA. Ah-receptor agonists generated from indole-3-carbinol *in-vitro* and *in-vivo*: Comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Proceedings of the National Academy of Sciences (USA). **88**:9543-9547.

Proposed Research
Christopher A. Bradfield

Background: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic and
carcinogenic compound which is widely dispersed in our environment. The risk associated
with exposure of human populations to TCDD continues to generate debate among the
scientific and regulatory communities. Our view is that a proper understanding of the hazards
posed by TCDD will only come from a thorough understanding of the mechanism by which
this chemical exerts its toxic effects. A large body of scientific evidence indicates that
TCDD-induced toxicity is the result of its binding to a soluble protein, known as the Ah-
receptor. The Ah-receptor appears to act as a TCDD-responsive transcription factor and is
known to regulate the expression of at least four genes, *CYP1A1* (encoding cytochrome
P450IA1), *Ya* (encoding the *Ya* subunit of glutathione s-transferase) and the genes encoding
tissue plasminogen activator inhibitor-2 and interleukin-1β. In addition, the Ah-receptor is
believed to regulate, additional, as yet undiscovered gene products involved in cell growth
and differentiation.

In our recent studies, partially funded by the Air Force Office of Scientific Research, we
purified the Ah-receptor to homogeneity, and microsequenced 4 internal peptide domains
generated by cyanogen bromide cleavage. Using a synthetic peptide approach, we generated
three sets of antisera which cross react with three unique epitopes of the Ah-receptor. We
have also generated large quantities of antisera against the most antigenic epitope in an effort
to provide a reproducible reagent for dissemination to the scientific community and for our
own research. The internal amino acid sequence data was also used to confirm the identity
and the reading frame of the cDNA encoding the Ah-receptor and provided localization of
the TCDD binding domain to the N-terminal third of the protein. Our recent results indicate
that the Ah-receptor belongs to a family of ligand responsive transcription factors with
related genes appearing in organisms as divergent as humans, mice, birds and drosophila.

Abstract and Specific Aims: We know that the panel of toxic effects and
sensitivity to TCDD can vary significantly from species to species. Even within a species,
different strains display marked differences in sensitivity. This phenomenon has made
estimating the risks associated with human exposure difficult for two reasons, 1) because it is
difficult to identify the appropriate animal model for use in extrapolation to humans and 2)
we don't know if all human populations respond in the same way. In addition to marked
differences in sensitivity to TCDD, different strains and species display marked
polymorphisms in Ah-receptor structure (e.g apparent molecular weight), as well as in their
affinity for ligand and for other cellular proteins. To better understand the relationship
between receptor polymorphism and TCDD toxicity, we propose the following specific aims.

Aim #1: Characterization of Ah-Receptor-Protein interactions. We have
constructed plasmids for the expression of large quantities of Ah-receptor in *E. coli* and are
in the process of making similar constructs for eukaryotic expression. We propose to use
this recombinant receptor to identify proteins which interact with the Ah-receptor *in vivo*.
Three independent strategies will be employed. First, the recombinant receptor will be
utilized to generate antisera optimal for immunoprecipitation of receptor-protein complexes.
This antisera will then be used in coprecipitation or affinity chromatography experiments to

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purify the Ah-receptor-protein complexes from cell extracts. The associated proteins will either be identified by amino acid microsequencing and/or their cDNAs cloned. A second related approach will be to prepare a chromatography column which contains cross-linked receptor. This receptor column will then be used to isolate proteins form cell extracts which have an affinity for the receptor. As above, purified proteins which have an affinity for the Ah-receptor can be identified by microsequencing strategies commonly employed in our laboratory. Finally, we propose to use the recombinant receptor protein to screen cDNA expression libraries for proteins that interact with the Ah-receptor. To this end, we will radiolabel the recombinant receptor "hybridize" it to proteins expressed in phage plaques and identify those clones which present an autoradiographic signal. This strategy was successful in cloning the MAX gene and should prove successful here, if the interacting domains are processed properly in the recombinant proteins.

Aim #2: Characterization of Receptor Processing and its role in Polymorphism.
We propose to map functionally important receptor phosphorylation sites within the Ah-receptor, identify sites which are induced by agonist binding and determine if those sites are conserved among different receptor polymorphs. The protocol will be to metabolically label cultured hepatocytes with ^{32}P in the presence and absence of agonist. Cell extracts from both nuclei and cytosolic fractions will be immunoprecipitated and subjected to proteolysis by CNBr and/or trypsin (we have already determined CNBr map and can deduce the trypsin map from the cDNA and microsequencing). Once the phosphorylation sites are localized, we can use site directed mutagenesis of the cDNA and transient expression in hepatocytes to determine their importance in receptor function and whether alterations in these sites are related to receptor polymorphism.

Aim #3: Identification of Human populations polymorphic at the Ah-locus. One potentially serious mistake in assessing risk from dioxin exposure is the assumption that all human populations have the same sensitivity and exhibit the same toxic effects. We predict that human populations will exhibit significant polymorphism in the quantity and structure of the Ah-receptor, as well as in sensitivity to agonist. We base this prediction on the observation that of the six inbred mouse strains studied in our lab, their exist three different receptor isoforms. Each form displays a unique affinity for agonist as well for other cellular factors (as yet unidentified). A similar degree of polymorphism also appears to exist among rat outbred strains. To identify human polymorphs, we propose to analyze a series of transformed human lymphoblasts, tumor biopsy samples and human autopsy samples for a variety of indicators of receptor structure, function and quantity. Included in this panel will be estimates of receptor size on SDS-PAGE, ligand dissociation kinetics, and quantity of receptor per cell as determined by a double antibody ELIZA assay which we are now developing.